REGULATED PRODUCTS SAFETY ASSESSMENT

Safety Assessment on the Safety and Efficacy of an Additive of 25-Hydroxycholecalciferol (Bio D® 1.25%) as a Feed Additive for Poultry for Fattening, Other Poultry (Layers, Broiler, Breeders), Ornamental Birds and All Pigs (RP1335)

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An application was submitted to the Food Standards Agency (FSA) in November 2021 from Huvepharma NV ('the applicant') for the authorisation of 25-hydroxycholecalciferol (Bio D® 1.25%) as a feed additive under the category of 'nutritional' additives, functional group 'vitamins, pro-vitamins and chemically well-defined substances having similar effect'. The additive contains a minimum of 1.25% 25-hydroxycholecalciferol and seeks authorisation for a modification of its production process using Pseudonocardia autotrophica M301 (DSM 32858) as a production organism, and to be used at a maximum dose of: 100 µg/kg, 80 µg/kg, 50 µg/kg of complete feed with 12% moisture in poultry for fattening, other poultry and pigs, respectively; 50 µg/kg, 40 µg/kg, 25 µg/kg in drinking water in poultry for fattening, other poultry and pigs, respectively.

The FSA/FSS concluded the additive and production strain were correctly identified. It is not a dusty product, however the particle size distribution was not characterised fully. The additive is stable for 24 months at room temperature and acceptably stable in premixtures and feed. Stability during pelleting could not be determined. Homogeneity in feed was demonstrated. The presence of live cells from the production organism in the final form of the product could not be ruled out.

The FSA/FSS cannot conclude on the safety of the additive Bio D® 1.25% for the target species and consumers as currently formulated when produced by P. autotrophica M301 (DSM 32858).

The additive is not a skin irritant or sensitiser and is not an eye irritant. The additive has a low dusting potential which would limit exposure through inhalation, however, uncertainties remain on its particle size distribution. Conclusions could not be drawn on the systemic toxicity potential of the

additive, and the presence of live cells from the production strain in the final product could not be ruled out. These factors pose a concern for the safety for the user/worker.
The additive can be considered safe for the environment.
25-hydroxycholecalciferol can be considered efficacious for the target species at the proposed doses of inclusion. Conclusions on chickens for fattening can be extrapolated to ornamental birds.
This Safety Assessment represents the opinion of the FSA and FSS.

1. Introduction

The FSA/FSS have undertaken an assessment of a feed additive (Bio D® 1.25%, Huvepharma NV, Uitbreidingstraat 80, Berchem, Belgium, B2600), of 25-hydroxycholecalciferol, under Assimilated Regulation (EC) No 1831/ 2003 (EC, 2003) under the category of 'nutritional' additives, functional group 'vitamins, pro-vitamins and chemically well-defined substances having similar effect'.

The additive 25-hydroxycholecalciferol ($25OHD_3$) is currently authorised for its use in premixtures for chickens and turkeys for fattening (max. content of 0.1 mg/kg of complete feed with 12% moisture content), for other poultry (max. content of 0.08 mg/kg of complete feed with 12% moisture content) and pigs (max. content of 0.05mg/kg of complete feed with 12% moisture content). The application under evaluation is for:

- A new use of the additive, to be incorporated in drinking water at a maximum dose of 0.05 mg/L for fattening poultry, 0.025 mg/L for pigs and 0.04 mg/L in poultry for laying and breeding.
- A new use in ornamental birds at a maximum dose of 0.1 mg/kg of complete feed (12% moisture) and 0.05mg/L in drinking water.
- A modification for the use of *Pseudonocardia autotrophica* M301 (DSM 32858) as a production organism.

In line with Article 8 of 1831/2003, the FSA/FSS has considered whether the feed additive complies with the conditions laid down in Article 5, including: safety considerations for human, animal and environmental health; efficacy of the additive for its intended effect; potential impairment of the distinctive features of animal products. This, and the guidance put in place by EFSA for the evaluation of feed additive applications, has formed the basis and structure for the assessment.

This document outlines the conclusions of the FSA/FSS assessment on the identity, safety and efficacy of the additive, and represents the opinion of FSA/FSS for the request of a new authorisation.

Table 1. Table showing products included in this assessment

Title	Product type	Intended use/s	Intended species or categories of animals
25-Hydroxycholecalciferol	Feed additive	Nutritional additive	All growing poultry, all other poultry, all pigs, ornamental birds

2. Assessment

2.1. Section II: Identity, characterisation and conditions of use

The additive contains a minimum of 1.25% of the active substance 25-hydroxycholecalciferol. The applicant provided data from several batches supporting the identification values outlined below (Table 2):

Table 2. Identity table for the additive Bio D® 1.25%

Composition additive	
Bio D® 1.25% concentrate	25 – 62.5 %
Maltodextrin	37.5 - 75 %
Composition Bio D® 1.25% concentrate	
25-Hydroxycholecalciferol	1.32 - 1.37 % w/w
1a,25-dihydroxycholecalciferol	0.019 - 0.030 % w/w
Cholecalciferol	0.04 - 0.2 % % w/w
Any related sterol	5.9 - 6.4 % w/w
Total related sterols	20.7 - 22.4 % w/w
Residues of fermentation media	-
Loss on drying	1 - 5.3 %
Appearance	Light brown to brown powder
Chemical-physical specifications	
Bulk density	0.332 - 0.386 g/cm^3
Dusting potential	35 – 60 mg/m^3
Particle size distribution	< 13.21 µm – 10% < 58.72 µm – 50% < 214 µm – 90%
Impurities	
Arsenic	< 0.04 mg/kg
Lead	< 0.05 mg/kg
Cadmium	< 0.018 mg/kg
Mercury	< 0.005 mg/kg
Total Aflatoxins	< 1.5 µg/kg
Total coliforms	3.5 x 10^2 – 2.4 x 10^3 CFU/g
Total aerobic count	< 30 CFU/g
Yeasts and moulds	-
Enterobacteriaceae	Absent in 25g
Salmonella spp.	Absent in 25g
PCDD/Fs	0.125 ± 0.038 ng/kg
DLPCBs	0.121 ± 0.076 ng/kg
PCDD/Fs and DLPCBs	0.245 ± 0.154 ng/kg

There is significant variability in the proportion of concentrate to carrier in the final formulation of the additive. The proportion of vitamin D_3 in the form of 25-hydroxycholecalciferol was shown to be consistent across batches. The additive has limited water solubility.

The Stauber-Heubach method showed the additive is not very dusty, however the fraction of particles below 10 µm was not further characterised. The method for determining the particle size distribution of the final form of the additive was not disclosed.

The nature and proportion of fermentation media residue that remains in the Bio D® 1.25% concentrate was not specified. The applicant provided a morphological study as supportive evidence to evaluate the presence of viable cells of the production strain in the final product. It was carried out on three different batches, three samples each, for which the strain did not appear to grow in agar plates. This method is not sufficient to rule out the presence of viable cells of the production strain, which cannot therefore be ruled out. This is particularly relevant given the potentially large proportion of fermentation media that could form part of the final formulation of the additive.

The additive was shown to be stable for 24 months when stored at room temperature (25^{o}C, 60% RH). Stability ranged between 91.6% and 100% in mash and between 78.9% and 87.8% after storage at room temperature. Stability during the pelleting process was tested at 80^{o}C, but no retention time was given, so no conclusion could be drawn. In water, the additive was shown to be stable between 90-100% at 20-25^{o}C and between 79-83% at 3-8^{o}C. Homogeneity of the additive was demonstrated.

The proposed conditions of use of the additive are described in Table 3:

Table 3. Conditions of use of Bio D® 1.25% proposed by the Applicant

Complete feed (12% moisture)	Min-Max Age	Typical content	Maximum content	Withdrawal period
Poultry for fattening Ornamental birds	-	34.85 µg $25OHD_3$/kg	100 µg $25OHD_3$/kg	-
Poultry for laying and breeding	-	34.85 µg $25OHD_3$/kg	80 µg $25OHD_3$/kg	-
Pigs	-	25 µg $25OHD_3$/kg	50 µg $25OHD_3$/kg	-
Water	**Min-Max Age**	**Typical content**	**Maximum content**	**Withdrawal period**
Poultry for fattening Ornamental birds	-	11.6-17.5 µg $25OHD_3$/kg	50 µg $25OHD_3$/kg	-
Poultry for laying and breeding	-	11.6-17.5 µg $25OHD_3$/kg	40 µg $25OHD_3$/kg	-
Pigs	-	8.4–12.5 µg $25OHD_3$/kg	25 µg $25OHD_3$/kg	-
All vitamin D sources	**Min-Max Age**	**Typical content**	**Maximum content**	**Withdrawal period**
Poultry for fattening Ornamental birds	-	-	125 µg $25OHD_3$/kg	-
Poultry for laying and breeding	-	-	80 µg $25OHD_3$/kg	-
Pigs	-	-	50 µg $25OHD_3$/kg	-

2.1.1. Conclusions on Section II

The additive and production strain were correctly identified. It is not a dusty product, however the particle size distribution was not characterised fully.

The additive showed good shelf-life stability for 24 months and acceptable stability in premixtures and feed. Stability during the pelleting process could not be determined. Homogeneity in feed was demonstrated.

Based on the evidence presented, as well as the potentially high proportion of the fermentation residue remaining in the final product, the presence of live cells from the production organism cannot be ruled out.

2.2. Section III: Safety

2.2.1. Safety for the target species and safety for the consumer

To support the safety of the additive in the target species, the applicant submitted a literature review on safety in chickens and pigs and a tolerance study in chickens.

The studies included in the literature review were performed using an additive that is not identical to the one under assessment. Therefore, the literature review is not sufficient to support safety of the additive in pigs or chickens.

The tolerance study in chickens was performed using an additive sample produced by a different strain to that under assessment.

To support the safety of the additive for consumers, the applicant submitted several toxicological studies, namely an *in vitro* micronucleus test, an Ames test, and two sub-chronic oral toxicity studies (28 days and 90 days). These studies were performed using an additive sample produced by a different strain to that under assessment.

The FSA had requested further evidence that the additive under assessment and those used in the tolerance and toxicological studies were identical. The information provided by the applicant within the certificates of analysis and specifications of the product did not provide sufficient evidence to demonstrate toxicological equivalence of the strain under assessment and the strain used in the safety studies. It is therefore uncertain whether the results obtained in the safety studies would be the same if the studies were conducted using additive produced by the strain under assessment.

Based on this constraint, the tolerance and toxicological studies could not be considered as part of the evidence package to demonstrate the safety of the additive for consumers and the target species.

2.2.2. Safety for the user/worker

The applicant provided a series of studies to evaluate the safety of the additive for users:

- An *in vitro* eye irritation study was provided following OECD guideline 438. The study complied with OECD requirements and was conducted to GLP. The endpoint combinations of the study show that the additive belongs to the “no category” UN GHS classification. The additive is not an eye irritant.
- An *in vitro* skin irritation study was provided following OECD guideline 439. The study complied with OECD requirements and was conducted to GLP. The endpoint combinations of the study show that the additive belongs to the “no category” UN GHS classification. The additive is not a skin irritant.
- A local lymph node assay was carried out in mice following OECD guideline 429 to evaluate the skin sensitisation potential of the additive. The study complied with OECD requirements and was

conducted to GLP. The lack of significant increased lymphoproliferation and lack of significant dose-related response showed the additive is not a skin sensitiser.

The FSA/FSS cannot conclude on the systemic toxicity potential of the additive given that the battery of toxicological tests was carried out with a different production strain to the one under assessment. Furthermore, the presence of live cells of the production strain in the final product could not be ruled out, and this could pose a risk to users/workers. The low dusting potential of the additive is likely to limit user exposure through inhalation, but uncertainties remain on the particle size distribution of the final formulation of the additive.

2.2.3. Safety for the environment

Following the principles of Phase I of the Environmental Safety Assessment Guidance for feed additives, $25OHD_3$ is recognised as a naturally-occurring substance and is not expected to exceed the environmental concentration through its use in animal feed at the proposed inclusion rates. The additive can be considered to be safe for the environment.

2.2.4. Conclusions on safety

The FSA/FSS cannot conclude on the safety of the additive Bio D® 1.25% for the target species and consumers as currently formulated when produced by *P. autotrophica* M301 (DSM 32858).

The additive is not a skin irritant or sensitiser and is not an eye irritant. The additive has a low dusting potential which would limit exposure through inhalation, however, uncertainties remain on the particle size distribution. Conclusions could not be drawn on the systemic toxicity potential of the additive, and the potential for live cells from the production strain to remain in the final product could not be ruled out. These factors pose a concern for the safety for the user/worker.

The additive can be considered safe for the environment.

2.3. Section IV: Efficacy

According to Assimilated Regulation 429/2008 (EC, 2008), efficacy studies are not required for vitamins, pro-vitamins and chemically defined substances having similar effects that are already authorised as feed additives.

$25OHD_3$ is already authorised in chickens for fattening, turkeys for fattening, other poultry and pigs. Previous evaluations of the efficacy of the active compound (EFSA, 2005, 2009) concluded that $25OHD_3$ can be considered an efficacious way of delivering vitamin D_3 supplementation in the proposed species.

Conclusions from chickens for fattening can be extrapolated to ornamental birds, as per Guidance principles.

3. Analytical methods evaluation

Conclusions on the analytical methods are presented here as an extract from the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for 25-hydroxycholecalciferol (EURL-FA, 2022):

> "For the determination of 25-hydroxycholecalciferol in the feed additive preparation (Bio D® 1.25 %) and premixtures the Applicant submitted two single-laboratory validated and further verified methods based on reversed-phase high performance liquid chromatography (HPLC) coupled to spectrophotometric (UV) detection.
>
> The following performance characteristics were obtained in the frame of the validation and verification studies; for the feed additive: a relative standard deviation for repeatability (RSDr) ranging from 0.6 to 1.2 %; a relative standard deviation for intermediate precision (RSDip) ranging from 0.8 to 1.2 %; and a recovery rate (RRec) of 100 %; for premixtures: a RSDr ranging from 0.5 to 2.5 %; a RSDip ranging from 1.3 to 3.3 %; and a recovery rate (RRec) ranging from 98 to 100 %.
>
> For the determination of 25-hydroxycholecalciferol in feedingstuffs the Applicant submitted a single-laboratory validated and further verified method based on reversed-phase ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS).
>
> The following performance characteristics were obtained in the frame of the validation and verification studies for the determination of 25-hydroxycholecalciferol in feedingstuffs: a RSDr ranging from 2.1 to 5.4 %; a RSDip ranging from 2.1 to 10.0 %; a recovery rate (RRec) ranging from 96 to 108 %; and a limit of quantification (LOQ) of 0.005 mg of 25-hydroxycholecalciferol / kg feedingstuffs.

> Based on the performance characteristics presented the EURL recommends for the official control (i) the two above mentioned single-laboratory validated and further verified methods based on HPLC-UV for the determination of 25-hydroxycholecalciferol in the feed additive and premixtures; and (ii) the single-laboratory validated and further verified method based on UHPLC-MS/MS for the determination of 25-hydroxycholecalciferol in feedingstuffs.
>
> For the determination of 25-hydroxycholecalciferol in water the Applicant submitted a modified protocol of the above mentioned HPLC-UV method dedicated for the feed additive. This HPLC-UV method with a modified protocol was used in the frame of stability studies of the feed additive preparation in water. The Applicant analysed different type of water samples spiked with the feed additive preparation and RSDr of 9.2 % was derived for a mass fraction of 10 µg of 25-hydroxycholecalciferol / L water. In addition, a limit of detection (LOD) and a limit of quantification (LOQ) of 2.5 µg and 5.0 µg of 25-hydroxycholecalciferol / L water, respectively, were reported by the Applicant. Thus, the EURL considers that the Applicant demonstrated an extension of scope of the HPLC-UV method dedicated to the feed additive, to water.
>
> Based on the available performance characteristics presented the EURL recommends for the official control the above mentioned HPLC-UV method for the determination of 25-hydroxycholecalciferol in water."

FSA/FSS accepts the EURL analytical method evaluation reports. FSA/FSS determined the analytical methods proposed as appropriate for official controls for this feed additive.

4. Conclusions

The FSA/FSS concluded the additive and production strain were correctly identified. It is not a dusty product, however the particle size distribution was not characterised fully. The additive is stable for 24 months at room temperature and acceptably stable in premixtures and feed. Stability during pelleting could not be determined. Homogeneity in feed was demonstrated. The presence of live cells from the production organism in the final form of the product could not be ruled out.

The FSA/FSS cannot conclude on the safety of the additive Bio D® 1.25% for the target species and consumers as currently formulated when produced by *P. autotrophica* M301 (DSM 32858).

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The additive can be considered safe for the environment.

25-hydroxycholecalciferol can be considered efficacious for the target species at the proposed doses of inclusion. Conclusions on chickens for fattening can be extrapolated to ornamental birds.

Abbreviations

Acronym	Definition
$25OHD_3$	25-hydroxycholecalciferol
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
EURL	European Reference Laboratory
FSA	Food Standards Agency
FSS	Food Standards Scotland
GLP	Good Laboratory Practices
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
OECD	Organisation for Economic Co-operation and Development
PCDD	Polychlorinated dibenzo-p-dioxins
PCDF	Polychlorinated dibenzofurans
DLPCB	Dioxin-like polychlorinated biphenyls
R_{Rec}	Recovery rate
RH	Relative Humidity
RSD_{ip}	Relative standard deviations for intermediate precision
RSD_r	Relative standard deviations for repeatability
UHPLC	Ultra-high performance liquid chromatography
UN GHS	United Nations Globally Harmonized System of Classification and Labelling of Chemicals
UV	Spectrophotometric

References

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